

LETTER

Role of the Gene *Miniature* in *Drosophila* Wing MaturationOleksii O. Bilousov,^{1,2,3} Iryna A. Kozheritska,^{1*} and Vladimir L. Katanaev^{2,3,4*}¹Department of General and Molecular Genetics, Taras Shevchenko National University of Kyiv, Educational and Scientific Centre "Institute of Biology," Kyiv, Ukraine²Department of Pharmacology and Toxicology, University of Lausanne, Lausanne, Switzerland³Department of Biology, University of Konstanz, Universitätsstrasse 10, Konstanz, Germany⁴Institute of Protein Research, Russian Academy of Sciences, Pushchino, Russia

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Summary: *Miniature* is an extracellular zona pellucida domain-containing protein, required for flattening of pupal wing epithelia in *Drosophila*. Here, we show that *Miniature* also plays an important role in the post-eclosion wing maturation processes triggered by the neurohormone bursicon. Wing expansion and epithelial apoptosis are drastically delayed in *miniature* loss-of-function mutants, and sped up upon overexpression of the protein in wings. *Miniature* acts upstream from the heterotrimeric Gs protein transducing the bursicon signal in wing epithelia. We propose that *Miniature* interacts with bursicon and regulates its diffusion through or stability within the wing tissue. *genesis* 50:525–533, 2012.

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Key words: *Drosophila*; *miniature*; wing maturation; apoptosis; epithelial-to-mesenchymal transition; bursicon

The wing of a newly emerged *Drosophila* fruit fly is a soft folded structure. Soon after fly eclosion from the pupal case, release of the neurohormone bursicon from the brain and its delivery to the wing by hemolymph trigger a number of processes in wing epithelial cells, collectively referred to as wing maturation (Honegger *et al.*, 2008). It includes epithelial-to-mesenchymal transition (EMT) and apoptosis of wing epithelia (Kimura *et al.*, 2004; Natzle *et al.*, 2008), accompanied by pumping of the hemolymph in between the dorsal and ventral wing layers and out to remove the cell debris (Togel *et al.*, 2008). These processes result in unfolding and expansion of the wings. Additionally, adhesion of the two cuticle sheets, their sclerotization, and melanization take place, finally producing the flat firm flight organ of the adult insect (Johnson and Milner, 1987).

Binding of bursicon to the G protein-coupled receptor Rickets on wing epithelia is considered the cause of these cellular events (Baker and Truman, 2002). Rickets activates the heterotrimeric Gs protein (Kimura *et al.*, 2004; Luo *et al.*, 2005), producing GTP-charged G α s subunit and the G $\beta\gamma$ heterodimer (Gilman, 1987). G α s-GTP activates the cAMP-PKA pathway responsible for induction of apoptosis (Kimura *et al.*, 2004), while the G $\beta\gamma$ part appears to regulate the signaling branch controlling EMT and wing expansion (Katanayeva *et al.*, 2010). Additionally, Tissue Inhibitor of metalloproteinases, integrins, and β -catenin has been implicated in *Drosophila* wing maturation (Brower and Jaffe, 1989; Kiger *et al.*, 2007).

The X chromosome-localized *miniature* (*m*) gene is required for the late stages of pupal wing development (Roch *et al.*, 2003). Its role is conserved among *Drosophila* species, as mutations in this gene, both in *D. melanogaster* and *D. virilis*, result in about 1.5-fold reduced wing surface without decrease in cell number (Dobzhansky, 1929; Kozheritska *et al.*, 2004). Ultrastructural investigation revealed a defect in flattening of the

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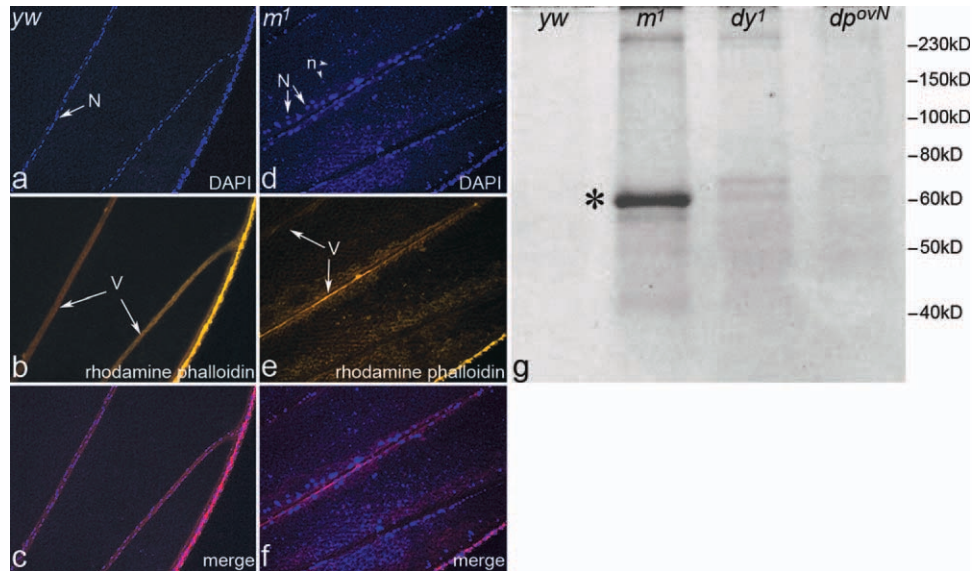


FIG. 1. Apoptosis and hemolymph removal are incomplete in *miniature* mutant wings. Wings of aged (>1d post-eclosion) *yw* (a–c) and *m¹* (d–f) flies were stained with DAPI (a, d) and rhodamine phalloidin (b, e). While control wings show surviving cells only along the veins and wing margin (a–c), adult *m¹* wings contain many remaining cells scattered across the wing blade (d–f). V: veins; N: nuclei; n: fragmented nuclei; g: analysis of the protein composition of *yw*, *m¹*, *dy¹*, and *dp^{ovN}* wings displays a dramatic increase in protein content in *miniature* mutant wings. The major band of the *m¹* wing extracts identified as apolipoprotein-2 is indicated with a star.

initially columnar wing epithelial cells, normally happening between 32 and 60 h after puparium formation, to be responsible for the overall reduced wing appearance in *miniature* mutants (Roch *et al.*, 2003). Additionally, incomplete adherence of the wing epithelial sheets and wing hair orientation defects have been described in *miniature* mutants (Newby *et al.*, 1991).

The protein encoded by the *miniature* gene has a signal peptide, a large extracellular portion with the zona pellucida (ZP)-domain and multiple glycosylation sites, a single transmembrane region, and a short intracellular tail. Since the transmembrane region is preceded by a furin-type endopeptidase cleavage site, the processed Miniature is assumed an extracellular protein (Jovine *et al.*, 2005; Roch *et al.*, 2003). The ZP-domain is an extracellular matrix (ECM) polymerization module and delineates a family of extracellular proteins with evolutionary conserved ECM organization functions (Jovine *et al.*, 2005). ECM proteins control numerous tissue activities, including regulation of diffusion of secreted signaling molecules such as morphogens and hormones (Hynes, 2009). Since *miniature* is strongly expressed in wing epithelia and affects the properties of the wing ECM (Ren *et al.*, 2005; Roch *et al.*, 2003), we questioned whether the wing maturation events triggered by bursicon might be regulated by Miniature.

Mature adult wings of *Drosophila* are mostly dead structures, with only vein and sensory cells remaining live (Fig. 1a–c). Cell debris could be seen in adult wings double mutant for *miniature* and its closely related ZP-

domain protein encoding gene *dusky* (Roch *et al.*, 2003), hinting at potential incompleteness of wing maturation in these mutants. Indeed, our analysis of the mutant line *m¹*, presumed null allele of *miniature* (Roch *et al.*, 2003), revealed that a significant amount of nuclear (DAPI) and cytoskeletal (rhodamine phalloidin) staining persisted in old (>1 day) *m¹* wings (Fig. 1d–f). DAPI staining of adult wings identified both intact and apparently fragmented cell nuclei (Fig. 1d). Thus, wing cell death appears incomplete in *m¹* animals.

To further characterize old (>1 day) *m¹* wings, we performed SDS-PAGE of their protein content, along with that of control wings. Despite the equal quantity of the source material and identical extraction conditions, much more protein could be extracted from *m¹* wings (Fig. 1g), in agreement with the persistence of cells and their debris in these wings. We performed similar protein extraction from wings of *dy¹* and *dp^{ovN}* mutants: loss-of-function alleles of *dusky*, which is involved jointly with *miniature* in flattening of the pupal wing epithelia (DiBartolomeis *et al.*, 2002; Roch *et al.*, 2003; Waddington, 1940), and *dumpy*, encoding another ZP-domain protein (Wilkin *et al.*, 2000), respectively. *dy¹* wings contained much less protein than *m¹*, while protein content of *dp^{ovN}* wings was essentially indistinguishable from wild-type.

By peptide mass-spectrometry, we identified the major protein band of the *m¹* wing extracts, migrating at 61 kDa (Fig. 1g), as apolipoprotein-2. This protein is secreted into the hemolymph by the fat bodies and it is one of the major hemolymph clot proteins (Karlsson

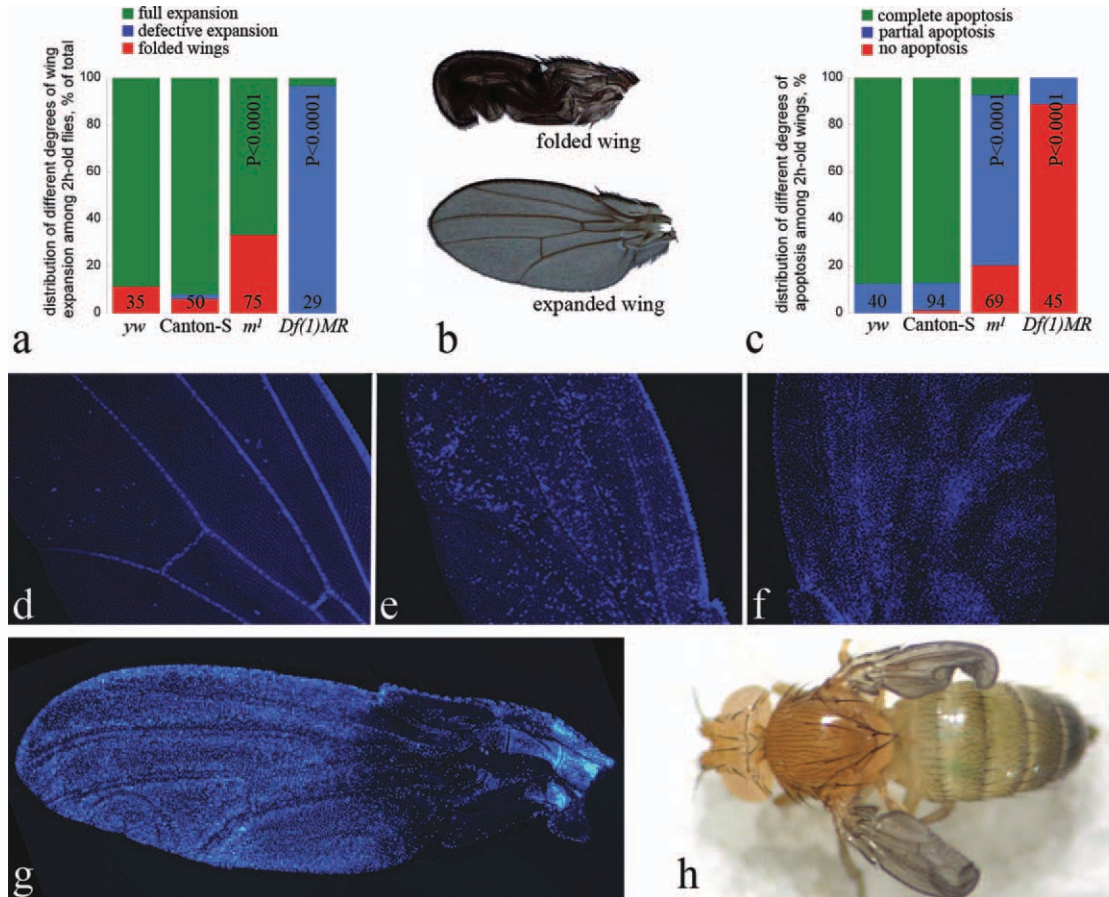


FIG. 2. Wing expansion and apoptosis are dramatically delayed in *miniature* mutants. (a) Quantification of distribution of different degrees of wing expansion (full expansion, defective expansion, and folded wings) among 2 h post-eclosion control (yw and Canton-S) and *miniature* mutant [*m¹* and *Df(1)MR*] flies. Numbers of flies analyzed is given at the basis of the bars. *P*-value of differences of the mutant genotypes from both control genotypes is from the Pearson's chi-squared test. (b) Examples of folded and expanded wings of the yw genotype. (c) Quantification of distribution of different degrees of apoptosis (complete, partial, or no apoptosis) among 2 h post-eclosion control (yw and Canton-S) and *miniature* mutant [*m¹* and *Df(1)MR*] wings. Numbers of wings analyzed is given at the basis of the bars. *P*-value of differences of the mutant genotypes from both control genotypes is from the Pearson's chi-squared test. (d–f): Examples of 2 h post-eclosion wings having complete apoptosis (yw, d), partial apoptosis (*m¹*, e), and no apoptosis (*m¹*, f). (g and h) *Df(1)MR* mutant animals show strongly enhanced phenotype of delayed apoptosis and at the same time incompleteness in wing expansion and flattening. (g) Most 2 h post-eclosion *Df(1)MR* flies have no apoptosis in wings. (h) *Df(1)MR* flies display defects in flattening their severely reduced wing. Wings in d–g are shown in scale.

et al., 2004; Kutty *et al.*, 1996; Scherfer *et al.*, 2004). Its presence in wings of the adult mutant flies implies that hemolymph is not properly flushed off the *m¹* wings and clots there.

To analyze whether wing maturation processes are indeed delayed in *miniature* mutants, we analyzed *m¹* and *m⁺* wings at 2 h post-eclosion, when EMT, apoptosis, and wing expansion are normally mostly completed (Johnson and Milner, 1987; Kiger *et al.*, 2007; Kimura *et al.*, 2004). Surprisingly, we found that 33% of *m¹* flies did not expand their wings by that time, as compared with only about 10% of the control animals (Fig. 2a,b). By the Pearson's chi-squared test, this difference is highly statistically significant ($P < 0.0001$). As wing expansion strongly correlates with EMT (Katanayeva

et al., 2010; Kiger *et al.*, 2007), we hypothesized that EMT is delayed in *m¹* flies. Relocalization of arm-GFP from cell boundaries to cytoplasm is typically used to monitor EMT in *Drosophila* wings (Kiger *et al.*, 2007; Natzle *et al.*, 2008). Unfortunately, strong cuticle auto-fluorescence of *m¹* wings (much stronger than in *m⁺* wings) prevented us from the direct investigation of EMT in this and other (see below) *miniature* mutant genetic backgrounds.

To monitor apoptosis, we performed DAPI staining of 2 h post-eclosion *m¹* and *m⁺* wings and found a striking delay in apoptosis in *m¹* flies (Fig. 2c–f). While about 90% of control wings have undergone complete apoptosis during the 2 h post-eclosion (Fig. 2c,d), intensive DAPI staining in *m¹* wings revealed that the vast major-

yw controls, as opposed to severely delayed apoptosis in m^D/Y and m^D/m^D flies (Table 1). This data may suggest that the mechanism of action of Miniature in the regulation of wing maturation is molecularly distinct from that in the earlier epithelial flattening program.

Fourth, not all *miniature* mutant combinations display the clear wing unfolding defect frequently seen in m^1 wings (Fig. 2a,b). Of the mutants studied, the following show strong penetrance (30–50% of flies) of this phenotype: $m^{259-4}/Df(1)MR$, $m^D/Df(1)BSC541$, $m^1/Df(1)BSC541$, and m^1/m^D (Table 1). In contrast, other mutants revealed 0–20% of 2 h post-eclosion flies with such defect. These frequencies are rather typical for the wild-type control flies (*yw* and Canton-S, Table 1). However, we note that some *miniature* mutants, not showing the clear wing unfolding defect as that of Figure 2b, still revealed incompleteness in wing expansion and flattening, such as shown on Figure 2h for a $Df(1)MR/Y$ fly. When such incomplete wing unfolding is taken into account, most *miniature* mutants analyzed corroborate with m^1 in terms of defective wing expansion (Table 1; Fig. 2a). Moreover, these phenotypes, as was found above for the delayed apoptosis, become more penetrant in the mutant backgrounds affecting both *miniature* and *dusky* genes (e.g., m^D/m^D , $Df(1)MR/Df(1)MR$, and $Df(1)MR/Df(1)BSC541$) where it reaches 80–100% of flies (Table 1; Fig. 2a). We propose, based on previous findings (Katanayeva *et al.*, 2010; Kiger *et al.*, 2007), that these wing expansion defects in *miniature* mutants reflect delayed EMT. Differences between the “choice” of the phenotype [such as folded wings in m^1 vs. incomplete flattening in m^D or $Df(1)MR$] may depend on other factors, for example, the stronger reduction in the wing size in the m^D and $Df(1)MR$ flies [see Fig. 2 and (Slatis and Willermet, 1954)]. Other, *miniature*-independent genetic factors may contribute to the penetrance of this phenotype (see below). In any respect, we stress that without direct EMT analysis the molecular significance of these wing expansion defects must be inferred with caution.

The above observations clearly demonstrate that Miniature is required for proper wing maturation. We next performed an epistasis experiment to roughly place Miniature in the hierarchy of the bursicon-initiated signaling steps leading to post-eclosion wing maturation. Expression of the GTPase-deficient (constitutively active) α -subunit of the heterotrimeric Gs protein ($G\alpha s[GTP]$) in wing tissue leads to precocious apoptosis; as a result, the dorsal and ventral wing sheets fail to adhere and hemolymph accumulates inside the wing producing blistered wings (Katanayeva *et al.*, 2010; Kimura *et al.*, 2004; Wolfgang *et al.*, 1996). Activation of the $G\alpha s$ -cAMP-PKA cascade is able to trigger apoptosis even when the neurohormone access to the wing epithelia is prevented by neck-ligation of pharate adults immediately after eclosion (Kimura *et al.*, 2004).

The dominant wing blistering phenotype of $G\alpha s[GTP]$ -expressing flies can be used to assess whether Miniature acts upstream of Gs (that is, at the level of bursicon or Rickets) or downstream of Gs (at the level of cAMP-PKA or cell death machinery) in the regulation of apoptosis. If Miniature acted downstream from Gs, $G\alpha s[GTP]$ would be unable to induce wing blistering in the m^1 genetic background (or its ability to induce blistering would be reduced). In contrast, if Miniature acted upstream from Gs, its removal would not affect the phenotype of $G\alpha s[GTP]$ expression.

We used *OK10-Gal4*, a wing-specific line driving expression weakly throughout the prospective wing blade at third larval stages and strongly throughout the wing blade at late pupal stages (Wolfgang *et al.*, 1996). As reported previously (Katanayeva *et al.*, 2010; Wolfgang *et al.*, 1996), expression of $G\alpha s[GTP]$ by *OK10-Gal4* leads to formation of blisters in 100% of flies (Fig. 3a). Importantly, the same was found in m^1 ; *OK10-Gal4*, *UAS-G\alpha s[GTP]* flies (Fig. 3b). Thus, we conclude that Miniature acts in the regulation of the wing maturation processes upstream from the heterotrimeric Gs protein.

Miniature is expressed by several cuticle-secreting tissues (Roch *et al.*, 2003) but not by most of other tissues (Chintapalli *et al.*, 2007; Fernandes *et al.*, 2010); of note, it is not detected in the nervous system where bursicon is produced (Honegger *et al.*, 2008). To prove further that the action of Miniature in post-eclosion wing maturation is wing-autonomous, we overexpressed *miniature* in wings using the *OK10-Gal4* driver. Since apoptosis is already mostly complete by 2 h post-eclosion in wild-type flies (Table 1), we compared flies 1 h post-eclosion without and with overexpression of *miniature*. Remarkably, we find that *OK10-Gal4*; *UAS-miniature* flies have markedly advanced apoptosis by 1 h post-eclosion (Fig. 3c,d, Table 2). At the same time, no blisters were ever seen in these flies, as opposed to those expressing $G\alpha s[GTP]$ in the same manner. Thus, it might be speculated that overexpression of *miniature* can speed-up bursicon-induced apoptosis, but does not induce precocious (bursicon-independent) apoptosis which is stimulated by activation of signaling at the level of $G\alpha s[GTP]$ or below (Katanayeva *et al.*, 2010; Kimura *et al.*, 2004; Wolfgang *et al.*, 1996).

We also tested other *Gal4* drivers to overexpress *miniature*, as well as different *UAS-miniature* insertion lines. Of the driver lines tested, *71B-Gal4* drives expression in the whole wing pouch excluding the margin (Capdevila and Guerrero, 1994), while *engrailed-Gal4* (*en-Gal4*) and *hedgehog-Gal4* drive expression only in the posterior half of the wing. Previously, such posterior expression of $G\alpha s[GTP]$ was found effective in inducing precocious cell death (Kimura *et al.*, 2004). We find that these *Gal4* driver

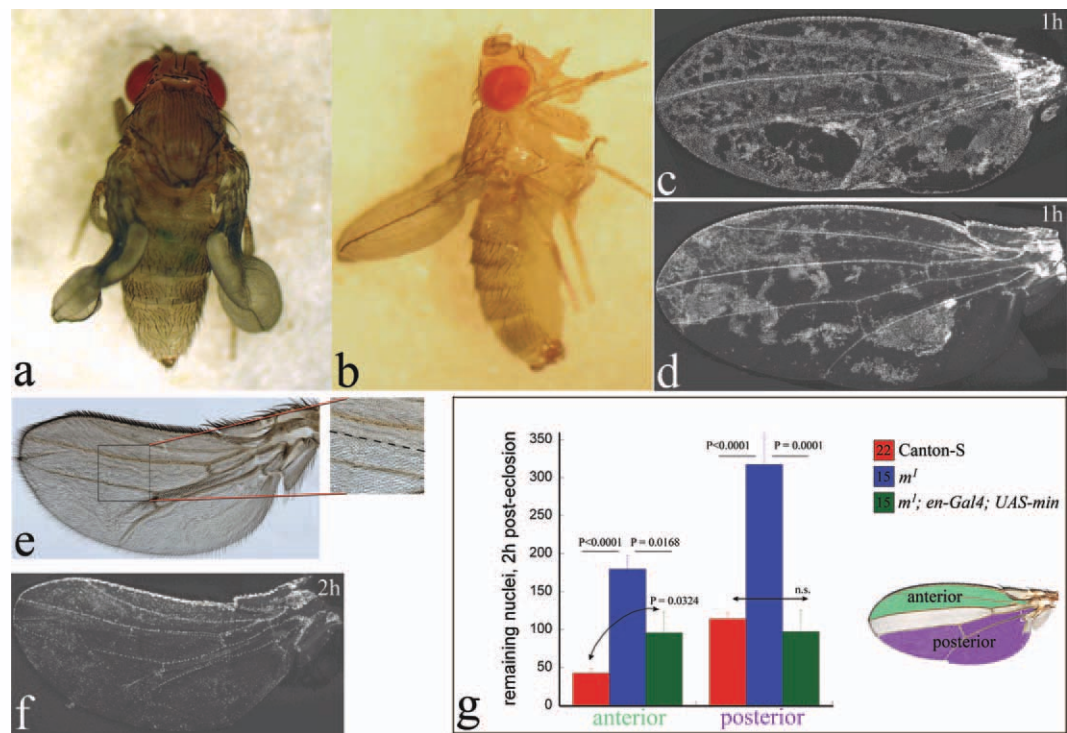


FIG. 3. Wing-expressed *miniature* functions upstream from the heterotrimeric Gs protein to regulate wing maturation in a nonautonomous manner. (a and b) Wing-specific expression of constitutively active G α s (*OK10-Gal4*, *UAS-G α s[GTP]*) results in wing blistering in 100% of pharate adults either wild-type (a) or mutant (b) for the *miniature* gene (*m*¹). (c and d) 1 h post-eclosion wild-type (Canton-S) wings have only begun their apoptosis (c), as opposed to *OK10-Gal4*; *UAS-miniature* flies which have markedly advanced apoptosis (d). (e and f) *en-Gal4*; *UAS-miniature* rescues *miniature* loss-of-function (*Df(1)MR*). While the rescue in overall wing size and wing cell cross-section dimension is strictly compartment specific (e, compartment border is shown with the dashed line on insert), apoptosis delay is efficiently rescued in both posterior and anterior compartments (f). (g) Quantification of surviving nuclei in 2 h post-eclosion wings of wild-type (Canton-S), mutant (*m*¹), and posteriorly rescued flies (*m*¹; *en-Gal4*; *UAS-miniature*) in the anterior and posterior compartments of the wings. Rescue in the posterior compartment is complete; rescue in the anterior compartment is partial but significant, revealing the nonautonomous action of *Miniature* in the wing. Insert shows the areas where quantification was performed. Numbers next to the genotype descriptions show the number of wings analyzed. *P*-values from the Student *t*-test show high statistical significance of differences between the genotypes; “n.s.”: non-significant (*P* > 0.05).

Table 2
Degree of Apoptosis in 1 h Post-Eclosion *UAS-Miniature* or Wild-Type Wings

	Canton-S	71B-Gal4	UAS-min; OK10-Gal4	UAS-min; 71B-Gal4	en-Gal4; UAS-min	UAS-min; hh-Gal4
Complete apoptosis	4.9%	11.5%	17.1%	24.4%	32.0%	35.7%
Partial apoptosis	21.3%	30.8%	65.9%	44.4%	32.0%	31.0%
No apoptosis	73.8%	57.7%	17.1%	31.1%	36.0%	33.3%
# Wings	61	26	41	45	25	42
<i>P</i> value		0.1153	<0.0001	<0.0001	<0.0001	<0.0001

Analysis was performed as in Table 1.

lines were all effective in speeding up apoptosis (Table 2). Interestingly, *UAS-miniature*; *71B-Gal4* flies also display more advanced wing expansion as compared to the parental *71B-Gal4* driver line in which wing expansion is retarded compared with other *Gal4* and wild-type lines: 42.9% of 1 h post-eclosion *71B-Gal4* flies have folded wings as opposed to 28.1% in *UAS-miniature*; *71B-Gal4* (*n* = 28–32 flies); this difference is highly statistically significant by the Pearson’s chi-squared test (*P* < 0.0001). Other *miniature* overex-

pression combinations were not different in their wing expansion from the parental *Gal4* lines.

Finally, we used one of these overexpression combinations (*en-Gal4*; *UAS-miniature*) to rescue *miniature* loss-of-function phenotypes [*Df(1)MR* and *m*¹]. Such posterior expression could clearly provide compartment-autonomous rescue of the cell size, resulting in an increase in the posterior wing area (Fig. 3e,f, and insert on Fig. 3e). We next tested whether the delay in wing maturation processes of *m*¹ and *Df(1)MR* flies would

Table 3
Degree of Apoptosis and Wing Expansion in 2 h Post-Ecdysis Miniature-Rescue Wings

	Canton-S	<i>en-Gal4</i>	<i>m¹</i>	Apoptosis <i>m¹; en-Gal4; UAS-min</i>	<i>Df(1)MR</i>	<i>Df(1)MR; en-Gal4; UAS-min</i>
Complete apoptosis	87.2%	95.8%	7.2%	87.0%	0.0%	21.4%
Partial apoptosis	11.7%	4.2%	72.5%	8.7%	11.1%	57.1%
No. apoptosis	1.1%	0.0%	20.3%	4.3%	88.9%	21.4%
# Wings	94	71	69	23	45	14
<i>P</i> value				<0.0001		<0.0001
	Canton-S	<i>en-Gal4</i>	<i>m¹</i>	Wing expansion <i>m¹; en-Gal4; UAS-min</i>	<i>Df(1)MR</i>	<i>Df(1)MR; en-Gal4; UAS-min</i>
Full wing expansion	92.0%	100.0%	66.7%	100.0%	3.4%	87.5%
Defective expansion	2.0%	0.0%	0.0%	0.0%	96.6%	0.0%
Folded wings	6.0%	0.0%	33.3%	0.0%	0.0%	12.5%
# Flies	50	39	75	12	29	8
<i>P</i> value				<0.0001		<0.0001

Analysis was performed as in Table 1.

be rescued by such expression. And indeed, both apoptosis and wing expansion defects of *m¹* and *Df(1)MR* were rescued to a large extent (Table 3; compare Fig. 3f with Fig. 2g). We were puzzled by the efficient rescue of the apoptosis phenotype in both the posterior and anterior compartments (Fig. 3f) which drastically differed from the rescue of the cell size, which was strictly compartment-autonomous. Thus, we analyzed more carefully the amounts of surviving nuclei in 2 h post-eclosion wings of wild-type, mutant, and posteriorly rescued flies in the anterior and posterior compartments. To avoid confusion at the border of the two compartments, we counted remaining nuclei in the part of the anterior compartment between wing margin and vein 3, while the posterior analysis was restricted to the area between vein 4 and the posterior margin (as schematized in insert of Fig. 3g). In this manner, more nuclei can be counted in the posterior region of 2 h post-eclosion Canton-S flies than in the anterior area (Fig. 3g). *m¹* mutants, as described above, show markedly increased numbers of nuclei in both areas. *en-Gal4; UAS-miniature* is found to completely rescue wing apoptosis to the wild-type levels in the posterior domain of the *m¹* wings (Fig. 3g). Remarkably, we find that the anterior region is also significantly rescued, although to the levels clearly below those of the posterior area. Altogether, these findings reveal a nonautonomous ability of Miniature to rescue wing maturation processes in *m¹* and *Df(1)MR* flies.

Components of the ECM can bind secreted molecules—hormones and morphogens—and regulate their diffusion, stability, and activity (Hynes, 2009). For example, type IV collagen directly binds Dpp, a *Drosophila* member of the TGF β family of secreted growth factors, to regulate its diffusion through developing tissues, contributing to Dpp signaling (Wang *et al.*, 2008). Similarly, ECM protein periostin expressed by stromal cells binds and accumulates Wnt ligands, which are critical for colonization of secondary tissues by metastatic cancer cells

(Malanchi *et al.*, 2012). We have found that a ZP-domain extracellular protein Miniature (and potentially its close homolog Dusky) is required for the post-eclosion maturation processes in *Drosophila* wings, which are initiated by the neurohormone bursicon. Miniature acts within the wing tissue, regulating the epithelial response to bursicon. Removal of Miniature markedly slows down apoptosis and potentially EMT in wing epithelia. Miniature acts upstream from the bursicon-transducing heterotrimeric Gs protein in these events and, unlike activated G α s, is unable to induce precocious apoptosis, although it does accelerate it upon overexpression. Finally, re-expression of Miniature rescues *miniature* mutant conditions to the full extent cell-autonomously, but also to a certain extent in a nonautonomous manner within the wing. Cumulatively, these findings speak for a function of Miniature in the regulation of diffusion/stability of bursicon through the pharate wing tissue, similarly to the examples of ECM-growth factor interactions described above. We propose that Miniature traps bursicon in the ECM, increasing its concentration or longevity of its presence for higher activation of the wing maturation processes. When re-expressed posteriorly in *miniature* mutants, the protein creates a posterior “sink” of the neurohormone, which can then diffuse anteriorly to trigger signaling also in this compartment.

Bursicon belongs to a large cysteine knot protein family of secreted factors and is mostly related to the vertebrate BMP (bone morphogenetic protein) antagonists such as Gremlin and Cerberus (Honegger *et al.*, 2008; Luo *et al.*, 2005; Vitt *et al.*, 2001), which play important roles in execution of BMP-dependent developmental programs (Walsh *et al.*, 2010). Similarly, the extracellular ZP-domain proteins are well conserved in animal evolution (Jovine *et al.*, 2005). We propose that Miniature directly regulates bursicon diffusion and activity in flies, and that their homologs may similarly interact in vertebrates.

MATERIALS AND METHODS

Fly Stocks and Crosses

The following *Drosophila* lines were used: *OK10-Gal4* and *UAS-Gxs/GTP* (Wolfgang *et al.*, 1996), *hh-Gal4* (Tanimoto *et al.*, 2000), *UAS-miniature* [insertion lines on chromosomes 2 and 3, (Fernandes *et al.*, 2010)], *Df(1)MR* (Roch *et al.*, 2003). Canton-S, *yw*, *dy¹*, *dp^{ovN}*, *m¹*, *m^D*, *m²⁵⁹⁻⁴*, *Df(1)BSC541*, *71B-Gal4*, *en-Gal4*, and *arm-GFP* were from the Bloomington *Drosophila* Stock Center. All crosses were performed at 25°C.

Protein Assays

Protein extraction was carried out from 50 wings of adult (>1 day-old) flies. Wings were frozen in liquid nitrogen and homogenized in SDS-PAGE buffer by 1 h-intensive vortexing with glass beads, followed by 0.5 h incubation at 55°C. Before applying to 10% SDS-PAGE, the samples were additionally boiled for 10 min. Four independent preparations with identical results were conducted. In-gel trypsin digestion and mass-spectrometry was performed by the Proteomics Facility of the University of Konstanz (Germany).

Microscopy

Whole flies were photographed through a Carl Zeiss Stemi 2000 binocular using the Canon PowerShot G10 camera. Adult wings were mounted as described (Katanaev *et al.*, 2005). For fluorescence labeling, wings were fixed in 4%-formaldehyde/0.2% Tween-20 in PBS (20 min RT), washed three times in 0.2% Tween-20/PBS at RT, dehydrated in chloroform (1 h; 17°C), dechitinized in heptane (3 h; 17°C), and washed again in 0.2% Tween-20/PBS (3 h; 17°C) before overnight staining with DAPI (1:10,000 dilution, Sigma) and 0.5 µM rhodamine phalloidin (Invitrogen) at 4°C. Excess of the dyes was removed with three washings prior to mounting the wings in Vectashield for fluorescence microscopy (AxioImager, Carl Zeiss). Analysis of the degree of apoptosis was qualitative (except for Fig. 3g), based on investigation of overall several hundreds of DAPI-stained wings, which could be easily grouped into those depicting “no apoptosis” (whole wing blade area filled with nuclei, with DAPI-negative cell outlines corresponding to no more than roughly 20% of the area), “complete apoptosis” (whole wing area devoid of nuclei, with DAPI-positive cells corresponding to no more than roughly 20% of the area), and the intermediate “partial apoptosis.” Fully folded wings were excluded from the apoptosis analysis due to difficulty in visualization.

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